Endotoxin Enhancement as a Possible Etiology of Early-Onset Group B Beta-Hemolytic Streptococcal Sepsis in the Newborn

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Group B streptococcal cells, either viable or heat-killed, contain a substance that induced fever in rabbits with maximal responses occurring four hours after intravenous injection. In contrast, supernatant fluids failed to induce significant fever. Group B streptococcal cells also enhanced host susceptibility to lethal shock by endotoxin as much as 40,000-fold. A graph of log streptococcal cell dose used for pretreatment versus log LD_{50} endotoxin gave a straight line with a slope of approximately −1. Rabbits that received both streptococcal cells and endotoxin showed initial fever followed by hypothermia, labored breathing, watery diarrhea, evidence of vascular collapse, and finally death. Animals that received streptococcal cells or endotoxin alone showed only fevers and mild diarrhea. A possible theory for the cause of death in the neonate infected with group B streptococci is presented. *(Obstet Gynecol 61:588, 1983)*

During the past 20 years, an early-onset infection syndrome associated with group B β-hemolytic streptococcus has emerged as a major cause of neonatal death. Attack rates as high as three to four per 1000 live births and mortality rates as high as 75% have been reported.[1] This early-onset infection syndrome usually appears within the first 48 hours of life, but can often be seen within the first 12 to 24 hours. As early as the 1960s, investigators noted a clinical similarity between early-onset group B streptococcal infection in the neonate and endotoxin shock in the adult.[2–4] Both may be characterized by respiratory distress, hypotension, temperature instability, leukopenia or leukocytosis, disseminated intravascular coagulation (DIC), oliguria, and failure to respond to appropriate antibiotic therapy.

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The possibility of pyrogenic exotoxin production by group B streptococci would not be unique among gram-positive cocci. Toxins of group A streptococci are thought to induce the clinical features of scarlet fever and may contribute to development of rheumatic fever and glomerulonephritis,[5,6] whereas staphylococcal enterotoxins produce numerous gastrointestinal symptoms. Recently, studies reported by Schlievert et al[7] have suggested that staphylococcal pyrogenic exotoxins may be associated with endotoxin to cause toxic shock syndrome by enhancing host susceptibility to lethal shock.

Although several groups have reported the isolation of potentially pathogenic extracellular products,[8,9] production of a lethal toxin has not been demonstrated for group B streptococci. However, a situation similar to the interaction of staphylococcal pyrogenic exotoxin and endotoxin that is thought to cause toxic shock syndrome could also occur with group B streptococcus. Studies were therefore conducted to determine whether a product of group B streptococci could act synergistically with endotoxin to produce similar effects in an animal model.

Materials and Methods

Bacterial Isolates

A clinical isolate of group B β-hemolytic streptococcus was obtained from a neonate who had succumbed to early-onset group B streptococcal infection. The isolate was serotype III and was maintained in the lyophilized state in the presence of fresh defibrinated rabbit blood. Group B streptococci were grown in pyrogen-free dialyzable beef-heart medium[7] until stationary growth phase was achieved. Plate counts were performed to estimate the number of bacterial colony forming units

(CFUs) per milliliter; stationary phase concentration was found to be 5×10^8 CFU/ml.

Both live and killed cells were studied. In experiments involving killed cells, the organisms were destroyed by exposure to 65C for 30 minutes. Cell-free supernatant fluids were prepared by centrifugation and passage through 0.45 μ filters (Millipore Corporation, Freehold, NJ).

Animals

Young adult American Dutch belted rabbits weighing 1.0 to 1.7 kg were used. The animals were kept in quarantine for one week before use. Healthy rabbits were conditioned to a restraining device for at least one day before use, and indwelling rectal temperature probes were inserted one hour prior to use in tests for pyrogenic exotoxin.

Endotoxin

Endotoxin was derived from *Salmonella typhimurium* by the phenol–water method of Westphal and associates.[10] It was diluted in phosphate-buffered saline (0.005 M phosphate, 0.15 M NaCl, pH 7.0) for intravenous injection. The LD_{50} of endotoxin was determined to be approximately 400 μg/kg by the method of Reed and Muench.[11]

Staphylococcal Pyrogenic Exotoxin C

Staphylococcal pyrogenic exotoxin C was prepared from strains of *Staphylococcus aureus* isolated from patients with toxic shock syndrome by the isoelectric focusing technique of Schlievert et al.[7] Purified toxin was administered intravenously in normal saline.

Group A Streptococcal Cell Walls

Group A streptococcal strain $T25_3$ (T12 gl)[12] was cultured overnight in 100 ml beef-heart medium. The cells were collected by centrifugation (650 × *g*, 10 minutes), restored to ten ml with phosphate-buffered saline, and killed by incubation in a 65C waterbath for 30 minutes. After cooling, the killed cells were treated with hyaluronidase (10 mg) for one hour at 37C. The cells were then centrifuged (650 × *g*, ten minutes), resuspended in phosphate-buffered saline (pH 8.0) containing 1% trypsin (Difco Laboratories, Detroit, Mich), and incubated an additional hour at 37C. Finally, the cell wall preparation was washed three times with phosphate-buffered saline (650 × *g*, ten minutes) and suspended in phosphate-buffered saline to an absorbance of 1.50 at 540 nm, which corresponded to approximately 2×10^8 CFU/ml of viable organisms.

Pyrogenic Activity of Group B Streptococci

Group B β-hemolytic streptococci were grown in dialyzable beef-heart medium, and 1.0 ml/kg of either live cells, killed cells, or supernatant fluid was injected into test groups of four animals each. Fever responses were then monitored hourly.

To determine whether the pyrogenic response with group B streptococci was similar to that seen with other pyrogens, healthy rabbits were prepared and injected with known pyrogens and febrile responses were compared. Endotoxin (1 μg/kg) derived from *S typhimurium*, staphylococcal pyrogenic exotoxin type C (3 μg/kg), and cell walls (1 ml/kg) derived from group A streptococcal strain $T25_3$ (T12gl) were each injected into four animals per group and the febrile responses were determined.

Endotoxin Enhancement

To assess the capacity of group B streptococcal cells or supernatant fluid to enhance host susceptibility to lethal endotoxin shock, groups of four animals were initially prepared and treated with either live cells, killed cells, or supernatant fluid. Also, six animals were pretreated with 1.0 ml/kg stationary phase concentration of viable pyrogenic exotoxin-negative *S aureus* cells. Each animal then received 10 μg/kg of endotoxin via a marginal ear vein four hours after initial treatment and was observed for response.

LD_{50} Determination

The LD_{50} group B streptococci and endotoxin were determined by administering concentrations of killed organisms at 0 hours and of endotoxin at 4 hours. LD_{50} determinations were made using the technique of Reed and Muench.[11]

Results

Healthy rabbits who received 1.0 ml/kg of live cells or killed cells (5×10^8 CFU/ml) developed the characteristic pyrogenic exotoxin fever response (Figure 1). This response was maximal at 4 hours and then returned to normal. Minimal pyrogenic response occurred with injection of supernatant fluid from the organisms. All animals survived without apparent sequelae.

When compared with the fever response curves of endotoxin (*S typhimurium*), peptidoglycan (group A streptococcus), and pyrogenic exotoxin C (*S aureus*), the fever response of the group B streptococcal organisms most closely resembled that of the staphylococcal pyrogenic exotoxin C (Figure 2). Again, all animals survived without apparent sequelae.

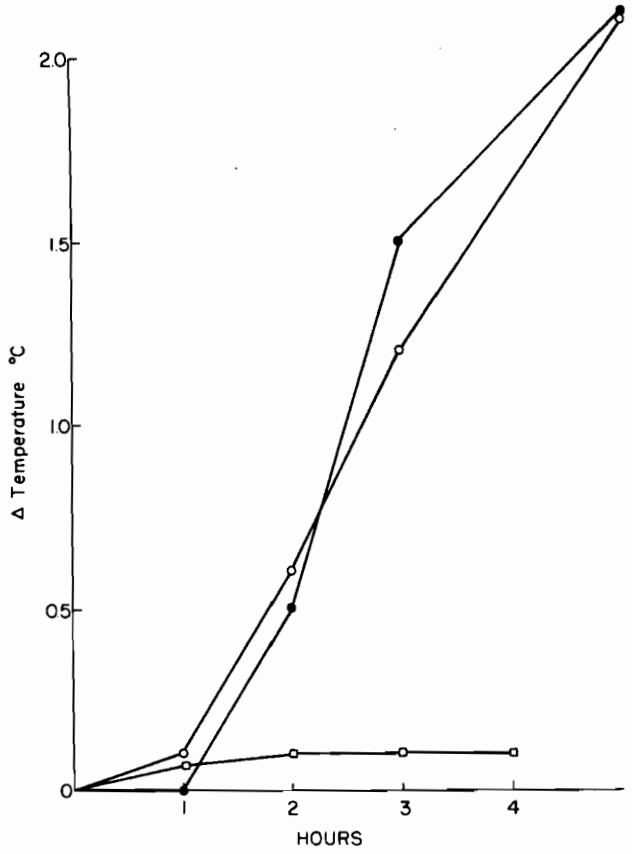

Figure 1. Pyrogenicity in rabbits of viable group B streptococcal cells (*closed circles*), heat-killed group B streptococcal cells (*open circles*), and cell-free culture supernatant fluids (*squares*). Group B streptococci cultured in dialyzable beef-heart medium until stationary phase growth (5×10^8 CFU/ml) achieved. All injections were intravenous (1 ml/kg) in phosphate-buffered saline; four rabbits per group. Average fever responses are shown.

As Figure 3 shows, all animals that were initially treated with the pyrogenic preparations of group B streptococci and four hours later received 10 μg/kg of endotoxin died. In contrast, no animals died when treated with the nonpyrogenic group B streptococcal preparation (supernatant fluid) and challenged four hours later with 10 μg/kg of endotoxin. Rabbits that received streptococcal cells immediately followed by endotoxin developed fevers followed by hypothermia, labored breathing, profuse watery diarrhea, evidence of vascular collapse, and finally death.

When rabbits were injected with live or killed cells or supernatant fluid concomitantly with 10 μg/kg of endotoxin, only fever and mild diarrhea occurred, and all animals survived. This was also true when the rabbits were pretreated with 10 μg/kg of endotoxin one hour before receiving either the cells or supernatant fluid. These findings suggest that endotoxin challenge causes death only when preceded by treatment with a pyrogenic preparation of group B streptococci.

By varying the number of killed organisms (given at 0 hours) and the concentration of endotoxin (given at 4 hours) administered to groups of four rabbits, the LD_{50} of each agent alone and in combination was determined. An inverse correlation between the two was found (Table 1). The concentration of endotoxin alone required to produce LD_{50} in the rabbits was found to be approximately 400 μg/kg. When the number of organisms was increased, the concentration of endotoxin required to achieve LD_{50} decreased. When 5×10^8/ml killed cells were used, the concentration of endotoxin required to achieve LD_{50} was 0.1 μg/kg, or 1/4000 the LD_{50} of endotoxin. The concentration of endotoxin needed to produce LD_{50} was 0.01 μg/kg when viable organisms grown to stationary phase (5×10^8) were used. This represents an endotoxin enhancement of at least 40,000 times. When the log concentration of killed cells was plotted against the log LD_{50} of endotoxin, a straight line with a slope of -1 resulted (Figure 4).

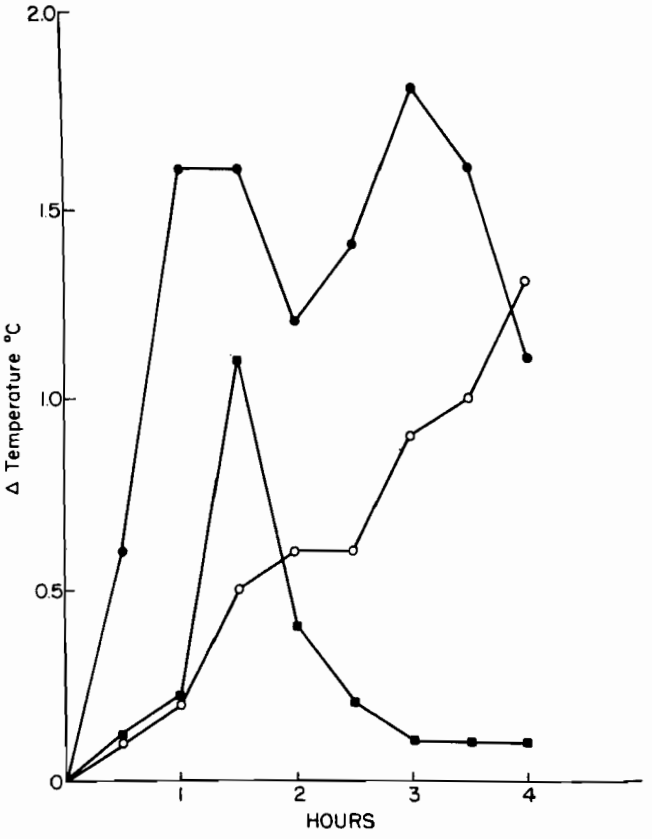

Figure 2. Pyrogenicity of endotoxin (*closed circles*), 1 μg/kg/ml, derived from *Salmonella typhimurium*, staphylococcal pyrogenic exotoxin type C (*open circles*), 3 μg/kg/ml, and cell walls from approximately 2×10 CFU/ml/kg of group A streptococci (*squares*). Endotoxin and group A streptococcal cell walls were administered intravenously in phosphate-buffered saline to groups of four rabbits; staphylococcal pyrogenic exotoxin was administered intravenously in normal saline to groups of four rabbits. Average fever responses are shown.

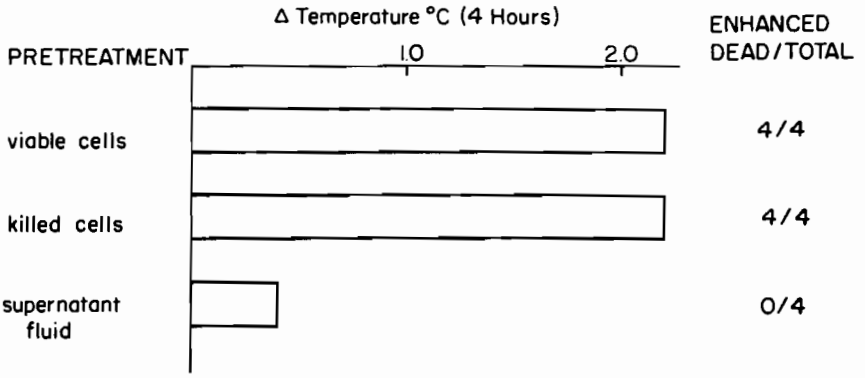

Figure 3. Capacity to enhance host susceptibility to endotoxin by viable or heat-killed group B streptococcal cells or cell-free culture supernatant fluids. Groups of four animals were pretreated for four hours with 5×10^8 CFU/kg/ml of viable or heat-killed cells or 1 ml/kg of culture fluid. Subsequently, each animal received 10 μg/kg/ml of endotoxin (1/50 LD_{50}) derived from *Salmonella typhimurium*. All injections were intravenous; endotoxin was administered in phosphate-buffered saline. Deaths were recorded over a 24-hour period.

Discussion

As early as 1966, Maher and Irwin[4] noted a clinical similarity between the early-onset infection syndrome in the neonate and endotoxin shock in the adult. Since these two conditions share many clinical features, an undefined group B streptococcal toxin may be either directly or indirectly responsible for the clinical features of the early-onset infection syndrome.

Pyrogenic exotoxins, which have been defined by their capacity to induce fever, also enhance host susceptibility to lethal shock by endotoxin.[5–7,13–16] Schlievert and associates have presented data[7,13] suggesting that the combination of staphylococcal pyrogenic exotoxin C and host-derived endotoxin may produce many features of toxic shock syndrome in rabbits. Although preliminary, the present authors' data suggest that a similar mechanism may operate with the group B β-hemolytic streptococcal strains employed in these experiments.

For each theory to be plausible, a source of endotoxin sufficient to produce the symptoms must be demonstrated. Bacterial colonization of the neonatal intestinal tract, including gram-negative bacteria, can occur as early as the first day of life.[17] Although dose susceptibility to endotoxin in the human newborn is not known, current knowledge suggests that the human is more sensitive than the rabbit to the effect of endotoxin.[12,18] Since prior treatment with viable group B streptococci decreased by 40,000 times the concentration of endotoxin required to kill 50% of the rabbits, a minimal amount of endotoxin would be required to produce an effect in humans.

The demonstration of an inverse relationship be-

Table 1. Enhanced Susceptibility of Healthy Rabbits to Endotoxin Shock by Group B Streptococci

Pretreatment	Results (dead/total): endotoxin (μg/kg) given four hours after GBS						Endotoxin LD_{50} (μg/kg)
	0.01	0.1	1.0	10.0	100	500	
None				0/4	1/4	2/4	400
With killed GBS cells							
1×10^6			0/3	0/3	2/3	3/3	58
1×10^7		0/3	0/3	1/3	3/3		18
1×10^8	0/3	1/3	1/3	3/3			1
5×10^8	1/3	1/3	3/3	3/3			0.1
With viable cells							
5×10^8	2/3	3/3	3/3	3/3			0.01

GBS = group B streptococci.

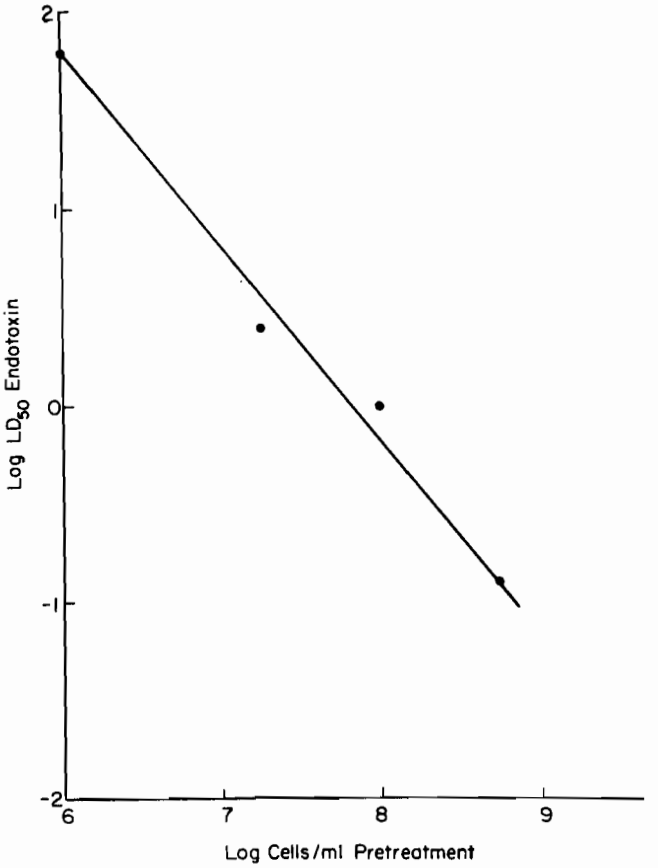

Figure 4. Quantitative relationship between heat-killed group B streptococcal cells and endotoxin in the enhancement of susceptibility to lethal shock in rabbits. Rabbits were pretreated with group B streptococci four hours before administration of endotoxin (from *Salmonella typhimurium*).

tween the log LD_{50} of endotoxin and the concentration of killed group B streptococci further suggests the presence of a pyrogenic toxin and is in agreement with previous reports of toxins from both group A streptococci[5] and toxigenic strains of *S aureus*.[13]

Hellerquist and associates[8] have recently reported the partial characterization of a carbohydrate–protein extracellular substance from a strain of group B streptococcus recovered from an infant with early-onset infection syndrome. The carbohydrate–protein was reported to have a molecular weight of approximately 200,000 daltons and caused increased pulmonary artery pressure, decreased Po_2, chills, and fever, but not death, in the sheep. The relationship between this carbohydrate–protein and the substance reported here is unknown.

The fact that lethal endotoxin enhancement did not occur when nonpyrogenic supernatant fluid was used might suggest that the pyrogenic response alone was responsible for the observed endotoxin enhancement. However, endotoxin challenge following pretreatment with either endotoxin or nontoxigenic *S aureus* failed to cause comparable mortality, in spite of a febrile response in the rabbit. Therefore, the enhanced susceptibility to endotoxin shock does not appear to be due to the febrile response alone, but to a product of the group B streptococci.

In conclusion, data from the present study suggest that a strain of group B β-hemolytic streptococci isolated from a newborn with early-onset infection contains a substance that increases the susceptibility of the rabbit to endotoxin at least 40,000-fold. The nature of the substance is unknown, but from the febrile response it does not appear to be either endotoxin or peptidoglycan. Although preliminary, a possible theory for the cause of death in the neonate infected with group B streptococci is presented. Further studies to elucidate the nature of this substance are currently being conducted in the authors' laboratory.

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